

CONVERSION OF METHANE INTO METHANOL
USING METHANOTROPHS

A REPORT BY
GEMINI BIOCHEMICAL RESEARCH LTD.

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SUMMARY

The methane oxidizing bacterium produced by Gemini Biochemical Research was tested for its ability to accumulate methanol under a number of differing conditions.

Under the conditions used to grow the organism the insoluble form of the enzyme was produced. This form of methane monooxygenase is very sensitive to inhibition by potassium cyanide. This inhibitor is added to preparations of the enzyme to prevent the further oxidation of the initial product of the reaction, namely, methanol. Without the addition of the cyanide it is impossible to accumulate methanol since it is oxidized by the enzyme methanol dehydrogenase which is also present in the preparations.

Attempts to physically separate the two enzymes by differential centrifugation were unsuccessful.

Other short chain alkanes and alkenes were substrates for the enzyme and their products could be accumulated under similar conditions to those used for methanol accumulation. However, no detailed studies were done with these systems.

A complete review of methane oxidation by methanotrophs was undertaken.

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INTRODUCTION

1 Methanotrophs: History, Properties and Potential Industrial Applications

1.1 History

Methanotrophs, or methane oxidizing bacteria, are micro-organisms (bacteria, fungi or yeasts) which have the ability to grow on compounds which have no carbon-carbon bonds and also have the ability to assimilate carbon as formaldehyde and carbon dioxide. Examples of compounds which methanotrophs can grow on include methane, methanol, dimethyl ether and methylamine. In nature, methane, derived via anaerobic breakdown of organic matter, is the most abundant substrate for these organisms. The majority of these organisms can grow on methane whereas a small proportion (especially yeasts) can only grow on methanol. These latter organisms are called methylotrophs to differentiate them from true methanotrophs.

Although it has been known that methanotrophs exist in nature for the past 70 years, only four species had been well documented prior to 1970. The first methanotroph to be described was isolated by Sohngen (1906) and was named Bacillus methanicus. It was re-isolated and renamed Pseudomonas methanica by Dworkin and Foster (1956). The other species isolated prior to 1970 were Pseudomonas methanitrificans (Davis et al., 1964) Methanomonas methanooxidans (Brown et al., 1964) and Methylococcus capsulatus (Foster and Davis, 1966).

Whittenbury and Wilkinson then reported on the isolation of over 100 strains of methane oxidizing bacteria (Whittenbury, 1969; Whittenbury et al., 1970).

1.2 Occurrence

Methanotrophs play a key role in the cycling of carbon in the biosphere. Methane, formed by anaerobic breakdown of dead plant and animal matter, is oxidized back to carbon dioxide by the methanotrophs thus allowing the carbon to be re-utilized by higher organisms such as plants and animals. Because of this central role in the cycling of carbon these organisms are ubiquitous in nature, occurring in lakes, ponds, sea water, soils, coal mine waters and sewage sludge.

1.3 Metabolism and Properties

Obligate methanotrophs (i.e. those organisms which can only grow on a one carbon substrate) have the properties illustrated in Table 1. They can be divided into two subclasses dependant on the pathway which they utilize to assimilate formaldehyde. Type I methane oxidizing bacteria assimilate carbon via the ribulose mono-phosphate pathway (Kemp, 1974). Type II organisms utilize the serine pathway for formaldehyde assimilation. Type II methanotrophs have a complete citric acid cycle whereas Type I organisms lack α -keto-glutarate dehydrogenase. Most Type II and some Type I organisms fix atmospheric nitrogen. A more detailed discussion of these organisms is presented in the review by Hanson (1980) and the text by Anthony (1982).

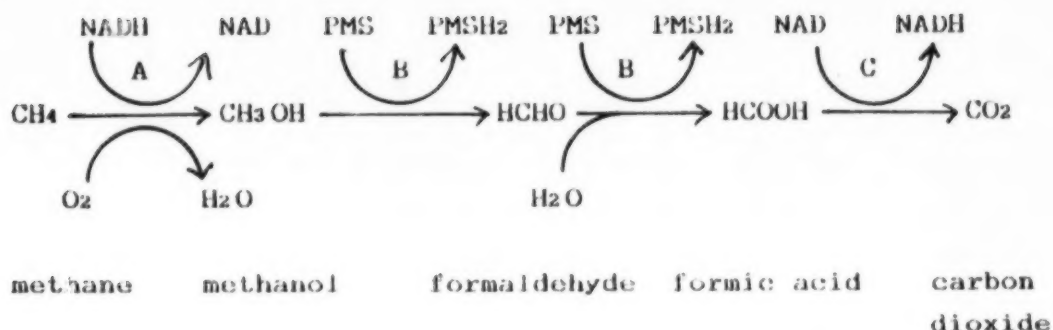
1.4 Methane Oxidation Pathway

Methane is oxidized to carbon dioxide in a series of two electron transfers. The pathway, as found in M. capsulatus, is illustrated below (Patel et al., 1972; Patel et al., 1973).

TABLE 1

Properties of Obligate Methanotrophs

1. They are all gram negative, strictly aerobic rods, vibrios or cocci.
2. They are catalase positive, oxidase positive and possess cytochromes of c, a or b type.
3. If nitrogen fixation is induced they become extremely oxygen sensitive.
4. They all use methane, methanol, dimethyl ether, methyl formate or methyl carbonate as sole source of carbon and energy.
5. They can use supplemental carbon sources such as propane, carbon dioxide or acetate. These compounds can only be co-oxidized and cannot be utilized alone.
6. They all use ammonia, most can use nitrate or nitrite.
7. They can all reduce nitrate to nitrite but will not grow anaerobically on methane since molecular oxygen is required for the initial oxidation of methane.
8. They all possess a complex arrangement of paired membranes
9. They all form a differentiated resting body (exospore or cyst).



Enzymes: A methane monooxygenase, B methanol dehydrogenase, C formate dehydrogenase

PMS phenazine methosulphate, NAD nicotinamide adenine dinucleotide

It should be noted that in this organism methanol is oxidized to formic acid by methanol dehydrogenase and that no separate formaldehyde dehydrogenase can be isolated. This is contrary to what has been found in *P. methanica* where a separate formaldehyde dehydrogenase has been detected (Ferenci *et al.*, 1975).

All the enzymes of the *M. capsulatus* pathway have been isolated and characterized with the exception of formate dehydrogenase which is quite unstable in cell-free extracts. For a detailed review of the properties of these enzymes reference should be made to the paper by Wolf and Higgins (1979).

1.5 Potential Industrial Applications

The potential industrial applications of methanotrophs fall into three general groups. These are:

- o Use of whole cells
- o Production of metabolic products
- o Miscellaneous uses such as fuel cells

1.5.1 Use of Whole Cells

a) Single Cell Protein (SCP)

Methanotrophs and methylotrophs both have very high growth rates thus making them suitable for the production of single cell protein. In addition, the cost of the substrate, methane or methanol is very low compared to other potential substrates. Bacterial cells produced on methanol or methane have protein contents upto 70% of the dry weight of the organisms. Yeasts have upto 50% of the cell mass as protein. Most large scale fermentations have chosen methanol (e.g. I.C.I.'s Pruteen plant and Phillips Petroleum's Provesta plant) because there is less heat generated during the metabolism of methanol than methane. Table 2 illustrates the oxygen demand and heat of fermentation for bacterial growth on a number of different substrates (Cooney and Levine, 1972). The removal of the heat of fermentation is a major expense in the production of single cell proteins. However, the use of thermophilic organisms (e.g. the methane oxidizers recently isolated from deep sea vents) will allow fermentations to run at higher temperatures and thus more cheaply. Another possibility is the use of recombinant DNA technology to confer heat stability to mesophilic organisms. Recently, two plasmids (Anon., 1983) of Bacillus stearothermophilus were transferred to B. subtilis. The resulting organism was found to carry the heat resistance characteristic of the donor.

A second reason for preferring methanol to methane is the potential for explosion hazards to exist during methane fermentations. However, by proper control of oxygen concentrations and gaseous phase recycle of unused methane, completely safe operation could be maintained (Hamer et al., 1967).

TABLE 2

Oxygen Demand and Heat of Fermentation for Bacteria Grown on Various Carbon Sources¹

<u>Carbon Source</u>	<u>Cell Yield²</u>	<u>Oxygen Demand³</u>	<u>Heat of Fermentation⁴</u>
Glucose	0.5	53	6.4
Methanol	0.5	150	18.0
Methane	0.6	560	67.0
n-Alkane	1.0	210	25.0

1. From Cooney and Levine, 1972.
2. g cells/g substrate.
3. mmoles/litre/hour.
4. Kcal/litre/hour.

The major drawback with these fermentations is that the product has to compete with alternate protein sources such as soybean meal and fish meal. Present pricing for substrates in the developed countries makes this fermentation non economical. The largest producer of single cell protein in the Western World, I.C.I., is presently investigating the possibility of licensing their technology to Arab states where the economics are more favourable.

There are a number of factors to consider when deciding which organism to produce. The major choice is to decide between yeasts or bacteria. Bacteria have higher growth rates and contain more protein (70%) than yeasts which are slower growing and have less protein (50%). However, yeasts, because of their larger cell size, are easier, hence less expensive, to harvest. In addition, yeasts may be more readily accepted by consumers because of familiarity (e.g. brewer's and baker's yeasts). Bacteria still have a negative image in the public's eye. It is difficult to determine a priori which is the best system since in the U.K., I.C.I. has been using a bacterium to produce its Pruteen and Philips Petroleum in the U.S. has used a yeast in its Provesteen process.

The future for single cell protein is still not clear. Additional product credits must be derived from the fermentation to make it profitable. Thus single cell protein must be coupled to the production of other products such as polymers, organic acids or vitamins. If the cells can be priced to reflect the vitamin and lipid content rather than the protein content then commercialization will be more profitable.

Vitamin content of methane grown cells is shown in Table 3.

TABLE 3

Vitamin Content of Various Products¹

<u>Vitamin</u>	<u>Dried Yeast</u>	<u>Skim Milk</u>	<u>Bacteria Grown on Methane</u>
(Concentration in mg/pound)			
Thiamine	43.0	1.5	9.0
Riboflavin	14.0	10.0	23.2
Niacin	180.0	5.7	79.8
Pantothenic acid	49.1	16.0	12.0
Choline	2000.0	3000.0	4840.0
Vitamin B ₁₂	0.0	0.3	4.8
Pyridoxine	25.2	0.0	71.8

1. From Kosaric and Zajic, 1974.

b) Demethanation of Coal Mines

Methanotrophic bacteria have the potential to oxidize the methane desorbing from coal. Field tests on the biological removal of methane have been conducted in at least two countries. In Russia, workers treated a longwall adjacent to a worked coal seam with fermentor grown cells of Methylococcus capsulatus (Ivanov et al., 1981).

Approximately 40% of the methane coming from the treated area was oxidized 10 days after application. In another test it was shown that Methylomonas methanica could oxidize all the methane in a coal seam after the cells were injected into the seam (Abramov et al., 1977).

Tests in India (Thakur et al., 1982) were also successful. In one test there was a 50% decrease in methane levels three days after nutrients and methane oxidizing bacteria were injected into a coal seam. In a second test there was complete removal of methane two months after similar treatment. Finally, in a third test the methane emission rate was reduced by approximately 66% twenty days after treatment. Unfortunately, the reports of these tests did not contain sufficient experimental data to determine if the conclusions were valid.

In addition to their use in coal seams, these organisms have the ability to oxidize methane in coal storage and transportation systems (Forrester and Thompson, 1983; Forrester and MacKeigan, 1984; Forrester and MacKeigan, 1986; Forrester and Richardson, 1987). These reports show that methane levels in these systems can be greatly reduced by the addition of cell cultures at the level of 3% (v/w). It is hoped that commercial scale testing can begin in 1988.

c) Petrochemical Transformations

The oxidation of methane to carbon dioxide by methane

oxidizing bacteria takes place in a series of two electron oxidation steps catalysed by the enzymes methane monooxygenase, methanol dehydrogenase, and formate dehydrogenase (section 1.4). Higher alkanes are not completely metabolized but are converted to the corresponding primary alcohols which are slowly converted to carboxylic acids. Secondary alcohols are oxidized to the corresponding methyl ketones.

Leadbetter and Foster (1958) showed that cell suspensions of Pseudomonas methanica could, in the presence of methane (to supply NADH), oxidize n propane and n-butane to their corresponding alcohols, aldehydes and acids.

The most complete study in this area has been undertaken by the microbiologists of Exxon Research and Engineering Company in New Jersey. This group has demonstrated the oxidation of C₃ to C₆ alkanes to their corresponding methyl ketones (Patel et al., 1980; Hou et al., 1981). Secondary alcohols were detected as intermediates in this reaction. The rates of production of the alcohols and ketones by a Methylosinus species and Methylococcus capsulatus are shown in Table 4.

A more interesting reaction was observed when alkanes were replaced by alkenes. The products of this reaction were the corresponding epoxides, valuable intermediates in petrochemical transformations. The rates of production of epoxides by three methanotrophs are shown in Table 5. The reactions were linear for approximately two hours, at which point they slowed down, presumably from lack of a co-factor.

Methanol grown or heat killed cells had no activity showing that the reaction was catalyzed by an inducible, labile enzyme system, methane monooxygenase. In additional experiments it was shown that enzymatically formed propylene

TABLE 4

Hydroxylation of Alkanes by Soluble Methane Mono-oxygenase
from Methylobacterium species¹

<u>Substrate</u>	<u>Product</u>	<u>Relative Activity</u>
Methane	Methanol	100
Ethane	Ethanol	69
Propane	1-Propanol	16
	2-Propanol	24
Butane	1-Butanol	27
	2-Butanol	46
Pentane	1-Pentanol	23
	2-Pentanol	48
Hexane	1-Hexanol	23
	2-Hexanol	42
Heptane	1-Heptanol	13
	2-Heptanol	54
Octane	1-Octanol	3
	2-Octanol	18
Isobutane	Isobutanol	37
	Tert. Butanol	43

1. From Patel and Hou, 1983.

TABLE 5

Oxygenation of Gaseous Alkenes by Cell Suspensions of
Methanotrophic Bacteria¹

Relative Reaction Rates

<u>Reaction</u>	<u>Methylosinus</u> <u>trichosporium</u>	<u>Methylococcus</u> <u>capsulatus</u>	<u>Methylobacterium</u> <u>organophilum</u>
Ethylene to Ethylene Oxide	35	100	16
Propylene to Propylene Oxide	65	100	45
1-Butene to 1,2-Epoxy- Butane	8	24	16
Butadiene to 1,2-Epoxy- Butene	45	80	51
Isobutene to 1,2-Epoxy- Isobutene	15	16	8

1. From Patel et al., 1982

oxide was not further metabolized and accumulated extracellularly.

Further work on this system is required in two areas before it can be economically viable. Firstly, any industrial process must include a co-factor regeneration step. Preliminary work in this area has recently been carried out (Patel *et al.*, 1983) where the co-factor, NADH, is regenerated by the addition of formate and formate dehydrogenase. Examples of this system are shown in Table 6. No indication was given as to how long this system could be operated. The reaction mechanism for the production of butylene oxide is shown in Table 7.

A simpler alternative may be to use a mixture of methane and the alkene.

Similar schemes can be designed for alcohol and methyl ketone formation.

The second area which needs to be improved is to develop a system where high concentrations of products can be obtained. One way to achieve this is to develop two phase systems where the product can be removed into a second phase and can accumulate there without interfering with the rate of production of the products (most of these reactions are inhibited when the product concentration reaches a certain level). Preliminary work in this area has recently been reported where epoxides reached 50% concentration in 1-octene (the 1-octene was both the substrate and the second phase in this process) (Smet *et al.*, 1983). In addition to a polar and non polar system, recent work has shown that products can be separated from cells in a two phase aqueous system (Brooks, 1983).

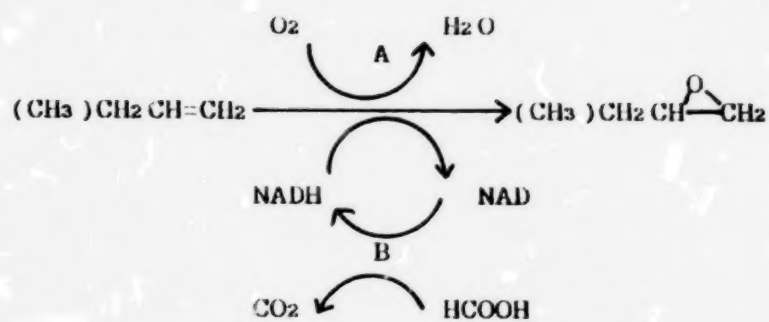
TABLE 6

Oxidation of Alkanes/Alkenes in Coupled Enzyme Systems for
Regeneration of NADH¹

<u>Substrate</u>	<u>Product</u>	<u>Cofactor Regenerating System</u>	<u>Relative Rate of Reaction</u>
Methane	Methanol		100
Ethane	Ethanol	NAD ⁺ -Linked	118
Ethylene	Ethylene Oxide	Formate Dehydrogenase	116
Propylene	Propylene Oxide		214
Methane	Methanol		121
Ethane	Ethanol		141
Ethylene	Ethylene Oxide	NAD ⁺ -Linked Secondary Alcohol	168
Propylene	Propylene Oxide	Dehydrogenase	232
2-Butanol	2-Butanone		232

1 From Patel and Hou, 1983.

TABLE 7



A: Methane monooxygenase
 B: Formate dehydrogenase

d) Production of Useful Products

Methanotrophs have been shown to produce a number of potentially useful products. The products can be classified into the following groups:

- o organic acids
- o vitamins
- o polymers
- o ammonia

Organic Acids

Citric acid is currently one of the fermentation products produced in the largest quantity worldwide. Traditionally, citric acid is produced by the fermentation of molasses in either stationary or submerged culture. Recently, however, methane and methanol have been suggested as feedstocks for this fermentation (Sisting, 1977). The organisms of choice for this fermentation would be the type II methanotrophs since they have a complete citric acid cycle.

Glutamic acid has recently been produced by methylotrophic organisms. Oki and Kitai (1974) showed that two strains of Methylomonas methylovario accumulated glutamic acid when they were grown on methanol. Maximum yields of 0.68% (w/v) in the fermentation broth were observed. The process has been patented by Aginomoto Company of Japan (Kono et al., 1972).

A methylotrophic butyric acid producing organism has been reported by Zeikus and his co-workers (Zeikus et al., 1980). The organism was named Butyribacterium methylotrophicum and it was able to carry out the anaerobic conversion of methanol (0.34%) into butyric acid (0.22%).

Vitamins

A number of workers have examined the production of vitamin B₁₂ by methylotrophic organisms (Nishio *et al.*, 1975; Kamibukubo *et al.*, 1978). The yields of soluble B₁₂ in the culture broths were rather low, 50-100 µg/litre, which is much lower than the values of 40 mg/litre found for *Streptomyces griseus* (Rickes *et al.*, 1948). If methylotrophs are to be used industrially for the production of B₁₂ higher yielding strains will have to be developed. It is more likely that whole cells will be used as a vitamin source since the cells contain high levels of B₁₂ and no expensive isolation procedures need to be used. The cells are also high in a number of other B vitamins (Table 3).

Polymers

Polymers produced by methanotrophic organisms can be divided into two classes, carbohydrate containing and non carbohydrate containing.

A number of carbohydrate containing bio polymers are being produced commercially. These include alginate and xanthan gum produced by Kelco and scleroglucan produced by CECA in France (for enhanced oil recovery). A number of other polymers have been examined and most have been shown to have useful properties. A number of reviews of industrially important bio-polymers have been published (Slodki and Cadmus, 1978; Kang and Cottrell, 1979; Sandford, 1979). The industrial applications for these polymers include: stabilizers, emulsifiers, or thickeners in food; mobility control in waterflooding of oil wells; plasma extenders; sizing of paper; paint, ink and cosmetic formulations; low oxygen permeable plastic films.

All of these polymers have been produced on carbohydrate containing growth media, however, a number of bio-polymers

produced on methanol have recently been described. The most extensively studied is the polymer produced by Methylobionas mucosa when it is grown in the presence of methanol (Tam and Finn, 1977; Finn et al., 1975; Finn et al., 1976). These workers showed that 57% of the methanol substrate could be converted into polymer in a nitrogen limited chemostat culture. This extra-cellular polymer greatly increased the viscosity of the culture broth. The viscosity of the polymer solution was not adversely affected by the addition of sodium chloride or by changing the pH of the solution (viscosity dropped off rapidly at pH values higher than 10). Potential applications for this polymer include: thickening agent for use in foods, cosmetics, paints, drilling muds, oil recovery; emulsifying agent, flocculant or deflocculant and soil conditioning agent (Finn et al., 1976).

A similar polymer was isolated from methanol grown cultures of Pseudomonas viscigena (Misaki, Tsumuraya and Kakuta, 1979). This polymer was unique in that it formed a water insoluble brittle gel in the presence of calcium ions.

Another heteropolysaccharide produced on methanol which had viscosity enhancing properties was isolated in the Laboratories of Exxon Research and Engineering Company (Hou, Laskin and Patel, 1978). This polymer was produced at concentrations of 1.4% (w/v) when Methylocystis parvus was cultured in the presence of 4% methanol. No potential uses were postulated, however, the polymer should have similar properties to other microbial polysaccharides.

Finally, the scleroglucan producers, Helotium species and Sclerotium rolfsii produced polymer when they were grown on 2% methanol (Gemini Biochemical Research Ltd., unpublished observations). Scleroglucans are produced commercially in Europe by CECA for use in enhanced oil recovery processes.

The only non carbohydrate containing polymer to be

considered in this section is poly-hydroxybutyrate (PHB). PHB was originally isolated as an energy storage polymer in organisms such as Alcaligenes eutrophus. ICI in the United Kingdom has isolated a strain which produces up to 80% of the dry weight of the cells as this intra cellular polymer when the organism is grown on carbohydrate containing media (King, 1982). The properties of PHB are very similar to polypropylene (see Table 8) with the added advantage that it is bio-degradable in soils and bio-erodable in animals. ICI has recently been issued a patent describing the production of PHB by the methylotrophic organism, Methylobacterium organophilum (Powell and Collison, 1982). In addition to growth on methanol, this organism could also grow on methane. The levels of PHB accumulating in the cells varied between 30 and 45% of the total dry cellular mass, corresponding to approximately 0.1% (w/v) concentration in the fermentor. Methanol concentrations were held at 1% (w/v) during the course of the fermentation.

Suggested uses for this polymer include medical uses such as degradable sutures, pins and other implanted devices as well as in formulations for controlled release of drugs and hormones.

2 Enzymology of the Methane Oxidation Pathway

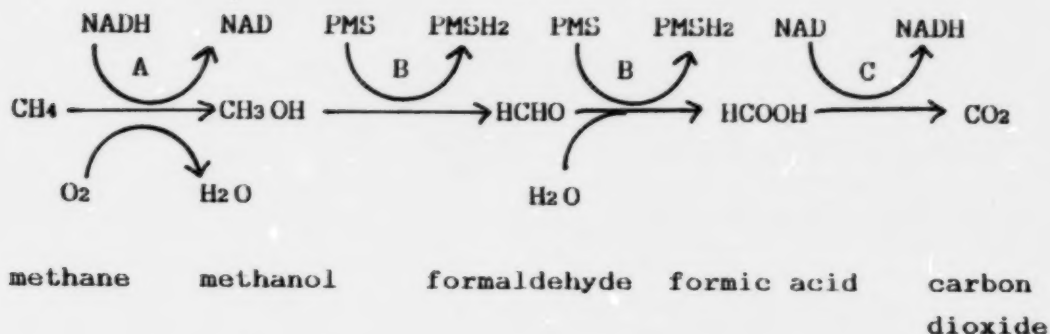
As mentioned previously, the microbial oxidation of methane occurs as a series of two electron transfers. This is shown below:

TABLE 8

Comparison of the Properties of Polyhydroxybutyrate and Polypropylene¹

<u>Property</u>	<u>PHB</u>	<u>PP</u>
Crystalline melting point (°C)	175	176
Crystallinity (%)	80	70
Molecular weight	5 x 10 ⁵	2 x 10 ⁵
Glass transition temperature (°C)	15	-10
Density (g/cm ³)	1.250	0.905
Flexural modulus (GPa)	4.0	1.7
Tensile strength (MPa)	40	38
Extension to break (%)	6	400
UV resistance	Good	Poor
Solvent resistance	Poor	Good
Biodegradable	Yes	No

1 From King, 1982



Enzymes: A methane monooxygenase, B methanol dehydrogenase, C formate dehydrogenase

PMS phenazine methosulphate, NAD nicotinamide adenine dinucleotide

The enzymes will be discussed in detail in the following sections.

2.1 Methane Monooxygenase

The first report on the metabolism of gaseous alkanes and an attempt to postulate a pathway for their metabolism was published in 1960 (Leadbetter and Foster). From experiments using whole washed cells of *Pseudomonas methanica* they showed that oxidation of alkanes occurred by oxidative attack at the terminal end of the alkane chain. Since *P. methanica* could not oxidize higher alkanes when they were added alone, methane was added as a co-metabolite. Corresponding alcohols and acids were isolated and identified from the reaction mixture. Thus ethane gave ethanol and acetic acid, propane gave n-propanol and propionic acid etc. When methane was added by itself the only detectable product was carbon dioxide.

Further work on this system was conducted by Brown *et al.*, (1964) who found that the addition of iodo-acetate, an enzyme inhibitor, caused an accumulation of small amounts of

methanol in similar experiments to those described above.

This finding could not be repeated by Higgins and Quayle (1970), however, they did find that the concentration of phosphate buffer in the reaction medium could influence the accumulation of methanol. In a series of experiments using labelled oxygen they showed that the enzyme responsible for the formation of methanol was a mono-oxygenase rather than an oxidase or a di-oxygenase.

Ribbons and Michalover (1970) attempted to localize the site of the methane mono-oxygenase in Methylococcus capsulatus. They showed that methane stimulated NADH oxidation was located in the cell fraction sedimenting between 3000 and 40000 g. This fraction contained most of the intracytoplasmic membranes which are so characteristic of methanotrophs.

Similar results were published by Ribbons (1975). This study also showed that in M. capsulatus the methane oxidizing activity was to be found in the particulate fraction. The only product which could be detected during this work was formate, i.e. no intermediates could be accumulated.

First reports on a soluble form of methane mono-oxygenase appeared in 1976. Dalton and his co-workers (Colby and Dalton, 1976; Colby et al., 1977) isolated and partially characterized a soluble form of methane mono-oxygenase from Methylococcus capsulatus. The soluble enzyme remained in the supernatant when cell free extracts were centrifuged at 160000 g for one hour. The enzyme had different properties from the enzymes isolated previously. The most distinguishing difference was its resistance to inhibition by cyanide ion and metal chelating agents. Purified preparations were shown to exhibit a broad substrate specificity. C₁-C₈ n-alkanes were oxidized to their corresponding alcohols and terminal alkenes were oxidized to

the corresponding 1,2-epoxides. In addition cyclic alkanes and aromatic compounds were hydroxylated.

The soluble enzyme was subsequently shown to consist of three components (Colby and Dalton, 1979):

- o component A, a large iron containing protein of molecular weight about 200000
- o component B, a small colourless protein of molecular weight about 15000
- o component C, a metalloflavoprotein of molecular weight about 45000

Component A is the site of hydrocarbon oxidation and oxygen activation. Component C is responsible for NADH binding and transfers two electrons to various acceptors, including protein A. The exact function of protein B is still unknown but it may be responsible for maintaining the correct three dimensional structure of the complex.

Very little is known about the structure of the insoluble form of the enzyme (Dalton and Higgins, 1987). Under denaturing SDS polyacrylamide electrophoresis three bands with molecular weights of 46000, 35000 and 25000 were observed.

However, major differences were noted in the enzymatic properties of the two enzymes. The most notable difference is that the soluble form is insensitive to cyanide inhibition whereas the particulate form is very strongly inhibited. A secondary difference is substrate specificity (Burrows *et al.*, 1984) in that the soluble form is much less specific and will oxidize a broader range of substrates as is shown in Table 9. In addition to substrate range there is also a difference in the products formed with the two

TABLE 9

Comparison of the Substrate Specificities of Soluble and Insoluble Methane Monooxygenase¹

<u>Substrate</u>	<u>Product</u>	<u>Reaction Rate with</u>	
		<u>Soluble MMO</u>	<u>Insoluble MMO</u>
Ethane	Ethanol	ND	ND
	Ethanal	25.0	35.0
Propane	Propanal	3.2	2.4
	Propan-1-ol	6.2	0.0
	Propan-2-ol	4.9	12.6
Butane	Butanal	2.6	0.6
	Butan-1-ol	4.2	0.0
	Butan-2-ol	7.3	5.9
Pentane	Pentanal	1.1	1.1
	Pentan-2-ol	0.3	0.5
Benzene	Phenol	74.0	0.0
Ethylbenzene	1-Phenylethanol	5.7	0.0
	4-Hydroxyethyl benzene	13.0	0.0
Styrene	Styrene oxide	82.0	0.0
Cyclohexane	Cyclohexanol	25.0	0.0

1. From Burrows *et al.*, 1984

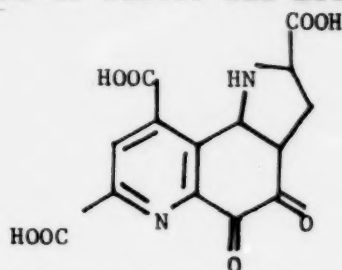
enzymes. The effects of various inhibitors on soluble and insoluble forms of methane monooxygenase are illustrated in Table 10.

Recently, two groups have tried to explain the controlling factors which govern which form of the enzyme will be expressed. Copper was shown to be one of these factors (Stanley, *et al.*, 1983; Burrows *et al.*, 1984). When the copper concentration in the growth medium was high (5 μ M) the particulate form was expressed. When the copper concentration was low (1 μ M) the soluble form was preferred. An earlier report suggested that oxygen availability may also be a factor since when oxygen was limiting growth the particulate form was expressed (Scott *et al.*, 1981).

2.2 Methanol Dehydrogenase

The first reports on an enzyme system capable of the oxidation of methanol were published by Anthony and Zatman (1964; 1967). These authors showed that a cell-free extract from methanol grown cells of *Pseudomonas* sp. M27 could oxidize methanol in the absence of NAD or NADP. Phenazine methosulphate was the only substance tested which could act as a hydrogen acceptor in the assay. The suggestion was made, based on spectrophotometric data, that the prosthetic group for the enzyme was a pteridine derivative.

However, later data obtained by X-ray analysis showed that the structure of the co-factor was methoxatin (Mincey *et al.*, 1981):



These authors also showed that their methanol dehydrogenase was inhibited by cyclo-propanol.

TABLE 10

Effect of Inhibitors on Soluble and Insoluble Methane
Monooxygenase¹

<u>Inhibitor²</u>	<u>% Activity</u>	
	<u>Soluble MMO</u>	<u>Insoluble MMO</u>
None	100	100
Potassium cyanide	100	0
2-Mercaptoethanol	100	0
2,2-Bipyridyl	99	10
Thiourea	90	0
Dithiothreitol	100	25
Imidazole	81	42
8-Hydroxyquinoline	29	0
Thioglycolate	100	40
Ethyne (3% in air)	0	0

1. From Dalton *et al.*, 1984.
2. Concentration of all inhibitors was 0.1 mM

2.3 Formaldehyde Oxidation

Two modes of formaldehyde oxidation have been established. In the first the enzyme methanol dehydrogenase can accept formaldehyde as an alternative substrate and oxidize it to formic acid. In the second route a separate enzyme, formaldehyde dehydrogenase is the enzyme involved. This enzyme utilizes NAD as a co-factor and is thus a source of NADH for the methane monooxygenase. Factors which determine which of the two enzymes are preferred for any particular organism are not well characterized and no further discussion will be made at this time.

2.4 Formate Dehydrogenase

The enzyme formate dehydrogenase is responsible for the oxidation of formate to carbon dioxide. A number of enzymes from various organisms have been studied and they all appear to be normal NAD dependant dehydrogenases. There has not been many extensive studies carried out on these systems (Anthony, 1982). This enzyme is a primary source of NADH for methane monooxygenase.

EXPERIMENTAL METHODS

1 Growth of Methane Oxidizing Bacteria

The strain of organism used in this phase of the work was the culture which had been adapted to grow in the small one litre fermentor described in a previous report (Forrester and Mackeigan, 1986). This organism had been cultured in a fed-batch mode for a period of six months. This strain was further adapted to grow in the bioreactor shown in Figure 1 and described below (Section 2).

The medium in which the organisms were cultured was Bushnell Haas medium consisting of the following ingredients:

Magnesium sulphate.....	0.2 g
Calcium chloride.....	0.02 g
Monopotassium phosphate.....	1.0 g
Dipotassium phosphate.....	1.0 g
Ammonium nitrate.....	1.0 g
Ferric chloride.....	0.05 g
Tap water.....	1000 ml

The bioreactor was operated in a fed-batch mode by replacing 80% of the growth medium with sterile fresh medium twice weekly. Air was sparged through the system at a rate of approximately 40 litres per minute. Methane was bled into the air supply at a sufficient rate to give a concentration of between 1 and 2% in the exit gas. The pH and temperature of the medium were not controlled during the operation of the bioreactor. Ambient temperature was approximately 20°C.

2 Description of the Bioreactor

The bioreactor was made from ABS plastic tubing. The main compartment in the bioreactor was 10 cm in diameter and was 2 m high. An external draft tube of 5 cm diameter, connected

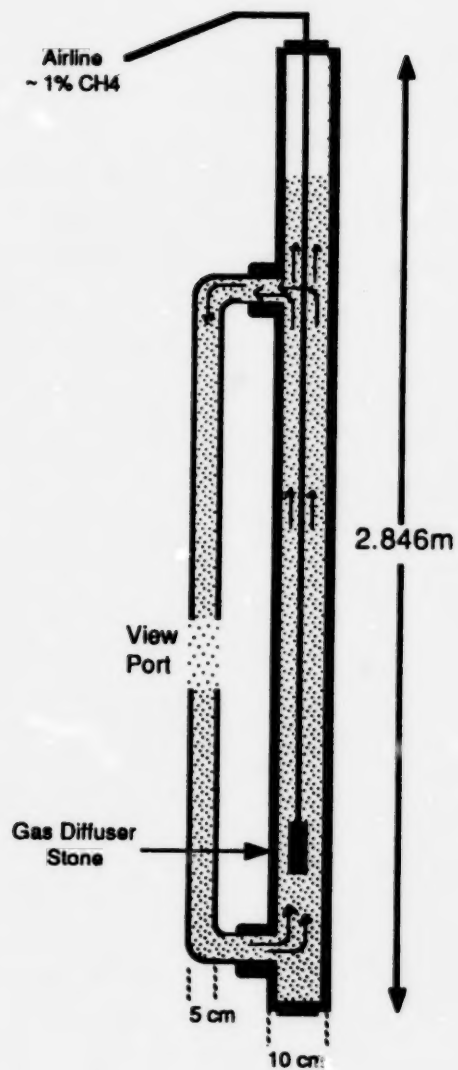


Figure 1.
Diagrammatic Representation of the Bioreactor

via 90° elbows and two "T" junctions, was fitted as shown in Figure 1. An additional piece of 12 cm tubing was attached above the top junction to prevent overflowing of the reactor when air was sparged through it. The total volume of the reactor was 30 litres but the nominal working volume was reduced to 25 litres due to gas holdup. Air was admitted to the reactor by a piece of tygon tubing which entered through the reactor top and was carried down inside the main compartment to a point approximately 15 cm above the bottom junction. A porous gas sparger was attached to the bottom of this tubing. A second tube exited from just inside the top of the reactor and was vented to the outside of the building for reasons of safety. Methane was bled into the air supply line and the flow rate adjusted so that methane concentrations in the exiting gas were kept within the range 1-2%(v/v).

It should be noted that this was not a sterilizable system but with proper care the bioreactor could be run in a satisfactory manner as discussed in the Results section.

Growth of the organisms was monitored daily by measuring the following parameters:

- (i) methane oxidation activity (see Section 4)
- (ii) optical density of the culture at 600 nm
- (iii) the dry weight of bacterial cells.

In addition, the pH of the culture was measured before each transfer.

3 Other Strains of Methane oxidizing Bacteria

In addition to the strain developed by Gemini Biochemical Research, the following strains were obtained from Torrey

Research Station in Aberdeen, Scotland:

- o Methylococcus capsulatus (Bath) NCIB 11132
- o Methylosinus trichosporium NCIB 11131

These cultures were grown in Bushnell Hass liquid medium in 250 ml Erlenmeyer flasks fitted with side arms. The flasks were sealed with a rubber membrane and the atmosphere was made 20% with methane. The flasks were incubated at 22° C on a rotatory shaker at 300 rpm.

4 Methane Oxidation Activity

Methane oxidizing activity of culture broths was measured as follows. Six ml aliquots of a cell suspension were added to 20 ml serum vials. The vials were capped with serum caps and 6 mls of methane were introduced into the vial via a syringe. The initial methane concentration was measured. The vials were incubated at 22°C on a rotatory shaker (300 rpm) for a period of five hours. A sample of the air space was removed with a syringe and the residual methane concentration measured. The difference between the initial and final methane concentrations was a measure of the methane oxidation rate of the culture broths. Carbon dioxide formation could also be measured during this incubation.

5 Whole Cell Methanol Synthesis

Cells obtained from fermentor or shake flask cultures were isolated by centrifugation and were resuspended in phosphate buffer. Reaction mixtures (1 ml) containing sodium phosphate buffer (50 μ moles) NADH (5 μ moles) and KCN (0.5 μ moles) were incubated in a methane atmosphere as described above for methane oxidation. Liquid samples were withdrawn via a micro-syringe at various times and assayed for methanol. Methane and carbon dioxide concentrations were measured at the start and end of the experiment.

6 Cell Free Methanol Synthesis

Cells were isolated and suspended in phosphate buffer as described above. The cells were sonicated at the maximum setting of a Microson Ultrasonic Cell Disrupter for 4 minutes. Care was taken to ensure that the cells were kept cold during this operation. The sonicate was centrifuged at 10000 x g for 10 minutes. Aliquots of both the pellet and supernatant were assayed for methanol synthesis as described above.

7 Other Gaseous Substrates

Reactions to test other substrates for methane mono oxygenase were carried out as described above for methane oxidation except that 6 mls of ethane, propane, propylene or butane were substituted for methane. Gas utilization and gaseous products (propylene oxide) were measured by removal of 3 ml aliquots of the gaseous atmosphere followed by analysis by gas chromatography. Liquid products were analyzed by gas chromatography as described for methanol synthesis.

8 Effects of Various Inhibitors

The following potential inhibitors for methanol dehydrogenase were tested at a number of varying concentrations:

- o 4 methyl pyrazole
- o iodoacetic acid.

The suicide inhibitors, cyclopropanone and cyclopropanol were not commercially available so they were not tested for their ability to cause accumulation of methanol.

9 Methanol Dehydrogenase Assay

Methanol dehydrogenase in sonicated extracts was assayed as described by Mincey et al. (1981).

10 Analytical Methods

Gases were assayed by gas chromatography using a Perkin Elmer Sigma 3 GC on a Supelco Carbosieve S 11 column (10' x 1/8"). Oven temperature was 225°C, detector temperature was 250°C. Carrier gas was helium at 30 ml/minute. Detection was by hot wire.

Methanol was assayed by gas chromatography using a Varian 1400 GC on a Poropak Q column. Oven temperature was 70°C and detector and injector temperatures were 250°C. Carrier gas was nitrogen at 25 ml/minute. Detection was by FID.

RESULTS

Detailed monthly progress reports (#'s 4, 5, 6, 7, 8 and 9) are included in Appendix A.

1 Methane Oxidation Activity

Figure 2 shows the methane oxidation activity of methane oxidizing bacteria grown in the 30 litre bioreactor. Maximum activity was obtained after a period of three days.

2 Methanol Synthesis and Accumulation

The results of a number of experiments to accumulate methanol are summarized in Table 11. As can be seen from the data the accumulation of methanol was unsuccessful. The enzyme system in these experiments was very sensitive to KCN and thus the further oxidation of methanol could not be prevented since levels of KCN which did not inhibit methane oxidation also did not inhibit methanol oxidation. Thus the only product which could be detected after oxidation of methane was the end product in the metabolic pathway, namely, carbon dioxide.

An experiment was conducted to measure the relative rates of synthesis and oxidation of methanol. This experiment showed that the methanol was oxidized 80 times faster than it was synthesised. This clearly shows why methanol does not accumulate in the absence of an inhibitor of methanol dehydrogenase.

3 Cell-Free Systems

Attempts were made to physically separate methanol formation from methanol oxidation. The results of this experiment are shown in Table 12. As can be seen both activities remained

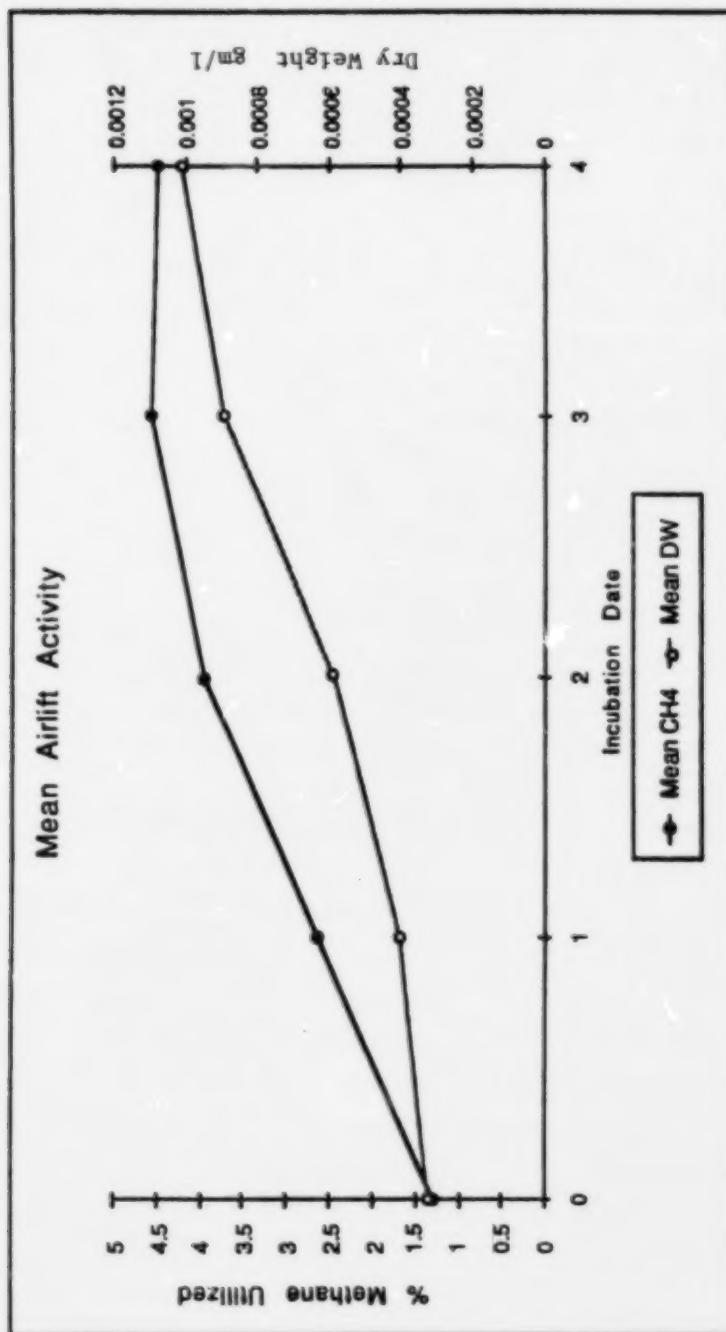


Figure 2
Bacterial Activity in the Bioreactor

TABLE 11

Summary of Attempts to Accumulate Methanol

<u>Concentration of KCN</u>	<u>CH₄</u> <u>Utilization</u>	<u>Methanol</u> <u>Accumulation</u>	<u>CO₂</u> <u>Formation</u>
0	Yes	No	Yes
0.0005 mM	Yes	No	Yes
0.005 mM	No	No	No
0.05 mM	No	No	No
0.5 mM ¹	No	No	No

1. Concentration used by Colby and Dalton, 1976.

TABLE 12

Location of Methane Monooxygenase and Methanol Dehydrogenase
in Cellfree Extracts

<u>Extract</u>	<u>MMO</u>	<u>MDH</u>
Whole Cell Homogenate	Present	Present
10000 x g pellet	Present	Present
10000 x g supernatant	Absent	Absent

in the insoluble portion of the cell extract and thus methanol was oxidized in these experiments.

4 Other Substrates

The methane mono oxygenase used in this study could accept a number of other substrates. Products from these substrates did accumulate under the conditions used in this study but since they did not fall under the conditions of the work statement further work on them was prohibited.

5 Other Strains of Methanotrophs

All strains of methanotrophs used in this study (see Experimental Methods) and grown under the same conditions produced the insoluble form of methane mono-oxygenase which was sensitive to inhibition by KCN.

DISCUSSION

A number of strains of methane oxidizing bacteria were tested for their ability to accumulate methanol. Unfortunately, under the conditions used for their growth only the insoluble form of the enzyme was expressed. This enzyme was as active as the soluble form but it was very sensitive to inhibition by KCN. As was described in the Introduction, two distinct forms of methane mono-oxygenase can be produced, a soluble form and an insoluble form. Initially, it was thought that the two forms were interconvertible but more recent work showed that they were two completely different protein complexes. In addition, the two forms have differing metabolic properties. The soluble form is insensitive to KCN and has a broad substrate specificity. The insoluble form is very sensitive to inhibition by KCN and has a much narrower substrate specificity.

Which form is expressed is determined by culture conditions. Low copper in the medium seems to promote formation of the soluble form whereas higher levels of copper favoured the insoluble form.

Another factor which appears to be involved is cell density. Lower cell density favours the insoluble form and high cell density favours the soluble form. In the experiments described here only low cell densities were obtained due to the culture conditions. In previous contracts with CANMET Gemini established conditions for producing cultures with high methane oxidizing activity. Unfortunately, these conditions also selected for the insoluble form of the enzyme.

A comparison of cell growth in this work with data obtained from other laboratories shows that the conditions used resulted in an average cell density between 1 and 2 mg/ml in

a period of three days. Other groups were able to get cell densities of between 7 and 10 mg/ml in periods of 20 days. Since we were interested in getting maximum activity in a short period of time our growth conditions resulted in the production of insoluble enzyme.

Recent data suggest that particulate methane monooxygenase is approximately 38% more efficient at converting methane into biomass than the soluble form (Dalton and Higgins, 1987). Thus conditions which are used to achieve maximum biomass accumulation (and methane oxidation rate) would select for the production of the particulate form of the enzyme.

Much more extensive experimentation than was available under this contract would have to be conducted if conditions for producing soluble enzyme are to be established in our laboratory.

The strain of organism used in this study appears to be very closely related to the new strain of methane oxidizing organism reported recently (Zhao and Hanson, 1984). Substrate specificity and inhibition characteristics of this organism and the one used in this study are very similar as can be seen from Table 13.

The methane mono-oxygenase used in this study did not have a broad substrate specificity but it could oxidize short chain alkanes and alkenes and the products of these reactions could accumulate under the conditions used. However, since these reactions did not fall under the scope of this project further study was prevented. The reason the products from these substrates accumulated was that the methanol dehydrogenase enzyme is much more specific than the methane mono-oxygenase, thus the products from the first reaction could not be further oxidized, or were only oxidized very slowly.

TABLE 13

Properties of Methane Monooxygenase in New Strain Isolated by Zhao and Hanson¹

<u>Substrate</u>	<u>Rate of Oxygen Consumption²</u>	<u>Products</u>
Methane	39	Carbon Dioxide
Ethane	23	Ethanol
Propane	42	2-Propanol 1-Propanol
Butane	2.2	2-Butanol 1-Butanol
Ethylene	5.2	Ethylene Oxide
Propylene	6.8	Propylene Oxide

1. From Zhao and Hanson, 1984
2. Nanomoles oxygen consumed per minute per mg of cells.

CONCLUSIONS

1. Conditions used to prepare methane oxidizing bacteria in our laboratory resulted in the production of the particulate form of methane monooxygenase.
2. Particulate methane monooxygenase prepared from these organisms was very sensitive to inhibition by potassium cyanide.
3. Methanol synthesized by particulate methane monooxygenase was further oxidized to carbon dioxide since methanol dehydrogenase could not be inhibited. Thus methanol could not be accumulated during this study. A number of different inhibitors were tested for their ability to block methanol oxidation without inhibiting methanol synthesis. Unfortunately, all three inhibitors tested did not have the necessary specificity to only block methanol degradation. All three also inhibited methanol synthesis. The more specific cyclo-propanol derivatives were not tested.
4. Other short chain alkanes and alkenes could act as substrates for particulate methane monooxygenase. Products from these substrates did accumulate but no further work was carried out with these systems.

RECOMMENDATIONS FOR FURTHER RESEARCH

1. Conditions for the production of soluble methane monooxygenase be studied.
2. Further experiments on the oxidation of short chain alkanes and alkenes by the particulate enzyme be conducted. This is potentially the most viable use for the industrial exploitation of methane monooxygenase. It is unfortunate that this study was not allowed to progress.

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Appendix A

Monthly Progress Reports #'s 4, 5, 6, 7, 8 and 9

Progress Report #4

Title of project: "Conversion of methane into methanol using methanotrophs."

(i) Free vs insoluble enzymes

A survey of recent research reports indicates that under the conditions we are growing the organisms i.e. high copper concentration (> 1ppm) the enzyme is totally in the insoluble form. This is further verified by the fact that our enzyme is inhibited by cyanide. This inhibitor only inhibits the insoluble form of the enzyme. Thus we will continue to work with the insoluble form for two reasons. Firstly, it will take considerable time to work out conditions for growing the organisms under copper deficient conditions. Secondly, the insoluble form of the enzyme is more stable than the soluble form, especially in crude extracts.

(ii) Inhibition of methanol dehydrogenase

Since the insoluble form of methane monooxygenase is inhibited by cyanide another inhibitor must be found. Two types of inhibitor may be used, 4-methyl pyrazole and the "suicide" inhibitor cyclo propanol. 4 Methyl pyrazole is available commercially so it was chosen for further study. Preliminary experiments showed that methane oxidation was inhibited if no NADH was present. The compound was shown to inhibit methanol dehydrogenase in sonicated extracts when it was added at the same concentration. One possibility is that the inhibitor is also inhibiting the MMO. The addition of NADH did not relieve the inhibition. This was unexpected so other electron acceptors will be tried e.g. succinate.

(iii) Other substrates for MMO

The oxidation of propylene will be examined. Propylene is a better substrate for examining what is happening since the primary product, propylene oxide is not oxidized by the methanol dehydrogenase. Thus the effects of the inhibitor 4 methyl pyrazole can be pinpointed more precisely. The propylene oxide accumulates and can be quantitated by gas chromatography. Preliminary experiments have shown propylene oxide formation when a mixture of methane and propylene is added to whole cells.

(iv) Experiments planned for the coming month

Further work on using propylene as a substrate will be carried out. The effects of the inhibitor 4- methyl pyrazole will be determined. The effectiveness of using succinate as an alternate electron acceptor will be studied.

Progress Report #5

Title of project: "The conversion of methane into methanol using methanotrophs".

(i) Literature search

An up-to-date computerized literature search has been undertaken. The literature search is up dated on a monthly basis. Key words which are searched include:

- o methane monooxygenase
- o methanol dehydrogenase
- o bacterial methane oxidation

The majority of the papers identified during this search have been obtained and catalogued. We are still awaiting receipt of a symposium volume on the chemistry of C₁ utilizing organisms. Once this has been received the literature search will be complete.

(ii) Screening of methanotrophs

Initial screening showed that the strains isolated in our laboratory are very similar. This was confirmed when testing of three strains showed that their methane oxidizing activities were identical in terms of quantity. One particular strain was adapted to grow in a small scale (1 litre) fermentor. This strain grew reproducibly and at a reasonable rate so all further work was carried out on that strain.

(iii) Isolation of methane monooxygenase

The experiments conducted in our laboratory have confirmed that the MMO produced by the strain of organism we are

growing is completely insoluble and membrane bound. The evidence for this is two fold. Firstly, sonication of the cells did not release any enzyme into the supernatant but was confined entirely to the 10000xg pellets. Secondly, the inhibitor profiles of the enzyme when determined on whole cells did not agree with the pattern established for the soluble enzyme. Specifically, it was shown that cyanide ion was an inhibitor of our enzyme while it did not affect the soluble enzyme as shown by other workers. Recent reports by Dalton and his coworkers have confirmed that the insoluble enzyme is inhibited by cyanide. They have also shown that the two enzymes (soluble and insoluble) are in fact different enzymes and cannot be interconverted. The ratio of soluble to insoluble depends on a number of factors including strain of organism used and the growth conditions.

The fact that our organism produced only the insoluble form of the enzyme has made this project more complicated than first envisioned. This is because in the original proposal we had planned to use the conditions for the soluble form i.e. we had planned to use cyanide as an inhibitor of the methanol dehydrogenase enzyme (the next enzyme in the pathway for methane oxidation). Unfortunately, as mentioned above, this is also an inhibitor of the insoluble methane monooxygenase. Thus an other inhibitor had to be found, or another substrate would have to be utilized, so that the product of the methane monooxygenase would not be further oxidized. The former route has been imposed upon us, therefore, we are examining the use of the inhibitor, 4-methyl pyrazole, which has been shown to inhibit methanol dehydrogenase. We should like to point out at this time that we feel that the better route would have been to utilize the alternative substrate (a well documented and preferred substrate by the majority of other workers) namely, propylene. However, we have adhered to the wishes of CANMET and are following the first route.

Since we are dealing with an insoluble enzyme it was felt that there was nothing to be gained by breaking open the cells so we have carried out very little of our experiments with sonicated extracts (except for the experiments where we showed that the enzyme was in fact insoluble).

Experiments have been carried out to determine the best buffer to use. Three buffers were chosen, phosphate, TRIS and TES. There was very little difference between the three buffers in terms of methane oxidation rates. However, the activity was shown to be more stable in the TES buffer than the other two. A pH profile in phosphate buffer was carried out. A pH profile on whole cells showed that methane oxidation activity had a broad peak between 6.0 and 8.5.

Experiments in which the concentration of phosphate buffer was varied showed that there appears to be a small accumulation of methanol at 60-80 mM phosphate. The small amounts of methanol were observed using the GC but concentrations were too low to allow for definitive identification. This work is being repeated on a larger scale so that the methanol can be concentrated for positive identification.

One of the problem areas is the source of electron acceptors. The soluble enzyme has been well shown to require only NADH. However, the situation is more complicated with the use of the insoluble enzyme. Ascorbic and succinic acids have both been reported to be used. We have compared NADH, ascorbic acid and succinic acid and could not see any stimulation of methane oxidizing activity by any of these compounds. In fact, we have shown that when the alternative substrates propylene and propane are utilized the respective products, propylene oxide and propanol (primary or secondary has not been determined at this point in time), are produced without the requirement for an electron acceptor. This may be because the products of the reaction (propylene oxide and

propanol) are rather poor substrates for the methanol dehydrogenase but enough of these compounds are oxidized to generate the in situ electron acceptor. In these experiments, preliminary estimates show that these substrates (propylene and propane) are converted in approximately 50% yields to their corresponding products. Results with two other substrates, methane and butane show zero accumulation of products. In the case of the product methanol, this product is further oxidized by the methanol dehydrogenase and does not accumulate to any appreciable concentration under the conditions used so far. With butane, there is no accumulation because the methane monooxygenase does not accept this compound as a substrate. This is in keeping with recently published data on the insoluble enzyme which showed that it only accepted hydrocarbons with a maximum chain length of 4-5 carbons whereas the soluble enzyme accepted substrates with chain lengths as long as 8-9.

Experiments in which we have attempted to inhibit the methanol dehydrogenase have not been successful. As mentioned previously, cyanide inhibits both the methane monooxygenase and the methanol dehydrogenase. The results with 4-methyl pyrazole have not been much better. Inhibition of methane oxidation at low concentrations was observed but no accumulation of methanol was found. More information on this system will be obtained when we test this inhibitor with propane as substrate.

(iv) Kinetic experiments

Work on these experiments will commence in the coming month. Propane will be used as a substrate since we have not been able to stop the reactions at methanol when methane is used. Large scale experiments will be carried out to determine which of the two possible propanols is formed. Work will continue with the experiments using the inhibitor, 4 methyl

pyrazole. Conditions will be varied to try and ascertain the optimum parameters to accumulate methanol as product. We have requested a culture of Methylococcus capsulatus and other methanotrophs which produce soluble methane monooxygenase from Dr. Dalton at the University of Warwick in England. Once this organism is obtained experiments will be set up to repeat the published work with this system.

Progress Report #6

Title of project: "The conversion of methane into methanol using methanotrophs".

(i) Literature search

The literature search is essentially complete. The symposium volume on the chemistry of C₁ utilizing organisms has been received. This volume contains an interesting paper on an organism with very similar properties to the one which we are using. The authors showed that this organism could accumulate alcohols when higher alkanes were used as substrates but did not accumulate methanol when methane was used. This is exactly what is happening with our system!

(ii) Kinetic experiments

Kinetic experiments were carried out using the following substrates:

methane
ethane
propane
butane.

The following results were obtained. With methane as substrate there was rapid oxidation of methane but no intermediate product (methanol) was detected. The results with ethane were essentially similar in that ethane was oxidized but no intermediates were detected. When propane was used a mixture of propanols was detected. The efficiency of conversion was estimated at between 75 and 80%. Under similar conditions butane was not oxidized.

(iii) Rate of methane oxidation vs methanol oxidation

Experiments were performed in which the rate of methanol oxidation was compared to the rate of methane oxidation. The results showed that the rate of methanol oxidation was much higher than the rate of methane oxidation, thus explaining why methanol does not accumulate. The rates were 37.5 μ moles/hour for methane and 937.5 μ moles/hour for methanol. The effects of 4-methyl pyrazole were measured. This inhibitor had no effect on the rate of methanol oxidized. Since this inhibitor was effective in cell-free preparation it is assumed that it cannot permeate through the cell walls of the whole bacteria.

(iv) Work to be carried out in the coming month

Conditions for the gas chromatograph will be adjusted so that we can separate the two propanol isomers so that the ratio of the two can be determined during the oxidation of propane. Experiments will be carried out with oxygen enriched air so that the concentration of methane and oxygen

can be increased during the oxidation reaction. This will speed up the rate of methane oxidation. Hopefully the rate will increase to such an extent that methanol will accumulate. If the culture of *Methylococcus capsulatus* is received from Dr. Dalton preliminary experiments using this organism will be performed.

Progress report #7

Title of Project: "The conversion of methane into methanol using methanotrophs".

(i) Literature search

The literature search is complete. A computerized check is made every month to obtain any information on new papers. During the month of August, Dr. Forrester attended the Fifth International Symposium on Microbial Growth on C1 Compounds. He had the opportunity to meet and discuss the work carried out on this project with other workers in the field. These discussions confirmed the results obtained, namely, that *Methylobacterium methanicum* only produces the insoluble form of the enzyme. Discussions with a microbial taxonomist resulted in the conclusion that our strain is most probably *M. methanicum*. Cultures of our organisms will be sent to him for identification.

(ii) Growth of other strains

Three strains of methanotroph have been received from the Torry Research Station in Aberdeen, Scotland. These strains are:

<i>Methylobacterium capsulatus</i> (Bath)	NCIB 11132
<i>Methylobacterium methanicum</i>	NCIB 11130
<i>Methylobacterium trichosporium</i>	NCIB 11131

These strains are now being cultured in our laboratory.

(iii) Inhibitor studies

Tests with iodoacetate were run to see if this inhibitor caused an accumulation of methanol. At low concentrations there was no effect and at higher concentrations methane oxidation was inhibited. Experiments previously carried out using cyanide as inhibitor were rerun. Results were as found initially i.e. methane oxidation was severely inhibited at low concentrations since the insoluble methane mono-oxygenase is very sensitive to this inhibitor.

(vi) Work to be carried out in the next few months

Work will commence immediately to test the effects of cyanide on the MOM from *M. capsulatus*. This should be a soluble enzyme and should be insensitive to this inhibitor. The inhibitor will inhibit the methanol dehydrogenase thus allowing accumulation of the methanol. Once conditions for small scale experiments have been established larger scale runs will be carried out after consultation with the Scientific Authority. The final report will be submitted by the end of December.

Progress Report #8

Title of Project: "The conversion of methane into methanol using methanotrophs."

(i) Other Methanotrophs

The following methanotrophs were obtained from Tory Research Station in Aberdeen, Scotland:

- o *Methylosinus trichosporium* (NCIB 11131)
- o *Methyococcus capsulatus* (NCIB 11132)
- o *Methylomonas methanica* (NCIB 11130)

These cultures were subcultured in small shakeflasks in low copper containing Bushnell Haas medium. *M. capsulatus* grew quite rapidly whereas *M. trichosporium* grows fairly slowly.

(ii) Methanol Production

M. capsulatus reacted in a very similar manner to the strains isolated by Gemini. The methane monooxygenase was very sensitive to cyanide and hence this inhibitor could not be used to inhibit the further oxidation of methanol as it was formed using whole cells. *M. trichosporium* appears to have greater potential. It was not as sensitive to the addition of cyanide and small quantities of methanol were detected using whole cells of this organism. However the accumulation of methanol (detected as a small peak on the gas chromatogram) was transitory and was oxidized during further incubation.

(iii) Cell Free Extracts

Work will be carried out on the cell-free methane monooxygenase obtained from *M. trichosporium*. The insoluble form of the enzyme should be relatively free of the methanol dehydrogenase, which should allow for accumulation of the methanol. Work has been slow over the past two months due to the slow rate of growth of *M. trichosporium* and other commitments, however, during the next two months much more work will be possible.

(iv) Final Report

The final report will be submitted on or before March 31, 1987.

Progress Report #9

Title of Project: "The conversion of methane into methanol using methanotrophs."

(i) Literature review

The literature review is complete. It is updated on a monthly basis.

(ii) Other methanotrophs

The two strains from Torry Station in Aberdeen were examined further. However, they exhibited the same properties as the strain which we ourselves have been using i.e. they produced the insoluble, cyanide sensitive form of methane mono-oxygenase, which cannot be used to accumulate methanol. The cells were disrupted by sonication but the methanol dehydrogenase remained in the insoluble fraction thus it was impossible to accumulate methanol with these preparations. Positive controls with propylene as substrate showed that the methane mono oxygenase had not been inactivated since the product, propylene oxide, accumulated under these conditions.

The insoluble fraction oxidized methane straight through to carbon dioxide suggesting that the methane oxidation pathway enzymes are linked in much the same fashion as certain fatty acid synthetases are.

This experiment has confirmed that copper concentration alone is not responsible for the transition from insoluble to soluble enzyme. Some other factor in the way in which we are growing the organism must be responsible for the transition.

(iv) New strains

Four new strains of methanotrophs were obtained from the Cranfield Biotechnology Centre in England. They are:

Methylosinus sporium 5
Methylosinus sporium 12 Type II
Methylocystis parvus OBBP
Methylomonas albus BG8 Type I

The first two are being reconstituted and will be grown in larger quantities and tested for soluble enzyme and cyanide sensitivity.